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ETIOLOGY AND RAPID DIAGNOSIS OF HUMAN VIRAL GASTROENTERITIS

Annual Report

Neil R. Blacklow, M.D.

May 01, 1987

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virus based on our detection of seroconversions to Norwalk virus in 12 of 20 patients experiencing gastroenteritis due to a strain of calicivirus. These two agents, therefore, may belong to the same family of viruses, as also may Snow Mountain agent for which we have also found seroconversions to Norwalk virus in some affected patients. In addition, these serological cross-reactions demonstrate the need for viral antigen specific detection methods such as we have previously developed for Norwalk virus. We have also confirmed the reported in vitro cultivation of 4 of 5 serotypes of human astrovirus, which is another agent that has been associated with some cases of gastroenteritis. Cultivation of astrovirus has provided sufficient antigen for us to demonstrate that astrovirus strains react type specifically when analysed by immunofluorescence but are group-reactive when tested by EIA. We have utilized this finding of an apparent astrovirus common group antigen to prepare and select monoclonal antibodies reactive against multiple astrovirus serotypes. These group reactive monoclonal antibodies now hold promise for facilitating epidemiological studies on the role of astroviruses in human viral gastroenteritis. Collaborative epidemiological studies are being done with the United States military on the role of enteric adenoviruses and Norwalk virus in overseas population groups, including troops on training maneuvers in which we have already shown Norwalk virus to cause outbreaks of diarrhea. We expect to expand our testing ability to include astroviruses and possibly caliciviruses in future studies.

SUMMARY

This project aims to develop means for the rapid diagnosis of etiologic agents of human viral gastroenteritis and to employ these means for an assessment of the epidemiological roles of these viruses in diarrheal disease. In addition, we are studying the immunological relationships of various gastroenteritis viruses to aid in classification and diagnosis of these agents. Efforts have concentrated on the development and utilization of immunoassays to detect etiologic agents, with the preparation and use of monoclonal antibody reagents where possible. We have prepared monoclonal antibodies specific for the enteric adenoviruses types 40 and 41, viruses thought to be of major importance in diarrhea. These antibodies have been characterized and used in an enzyme immunoassay (EIA) format to detect the enteric adenoviruses in 71 known positive diarrheal stool specimens with 95 to 98 per cent sensitivity and specificity. We are now employing this enteric adenovirus monoclonal antibody EIA to assess the epidemiology of this infection in several populations. We have shown that another gastroenteritis virus, calicivirus, shares antigenic characteristics with Norwalk virus based on our detection of seroconversions to Norwalk virus in 12 of 20 patients experiencing gastroenteritis due to a strain of calicivirus. These two agents, therefore, may belong to the same family of viruses, as also may Snow Mountain agent for which we have also found seroconversions to Norwalk virus in some affected patients. In addition, these serological cross-reactions demonstrate the need for viral antigen specific detection methods such as we have previously developed for Norwalk virus. We have also confirmed the reported in vitro cultivation of 4 of 5 serotypes of human astrovirus, which is another agent that has been associated with some cases of gastroenteritis. Cultivation of astrovirus has provided sufficient antigen for us to demonstrate that astrovirus strains react type specifically when analysed by immunofluorescence but are group-reactive when tested by EIA. We have utilized this finding of an apparent astrovirus common group antigen to prepare and select monoclonal antibodies reactive against multiple astrovirus serotypes. These group reactive monoclonal antibodies now hold promise for facilitating epidemiological studies on the role of astroviruses in human viral gastroenteritis. Collaborative epidemiological studies are being done with the United States military on the role of enteric adenoviruses and Norwalk virus in overseas population groups, including troops on training maneuvers in which we have already shown Norwalk virus to cause outbreaks of diarrhea. We expect to expand our testing ability to include astroviruses and possibly caliciviruses in future studies.

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

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BACKGROUND INFORMATION ON VIRAL GASTROENTERITIS

Acute viral gastroenteritis is an extremely common illness that affects all age groups and occurs in both epidemic and endemic forms (1). It is second in frequency only to the common cold among illnesses affecting United States families under epidemiological surveillance. It is also responsible for some of the common travelers' diarrhea encountered in Latin America, Africa, and Asia. The illness varies in its clinical presentation, but in general it begins with an explosive onset, and consists of varying disabling combinations of diarrhea, nausea, vomiting, low grade fever, abdominal cramps, headache, anorexia, myalgia, and malaise. It can be severe, indeed fatal, in the elderly, infant, debilitated, or malnourished patient.

Viral gastroenteritis occurs primarily in two epidemiologically distinct clinical forms (1). One entity is characteristically epidemic and is responsible for family and community-wide outbreaks of gastroenteritis among older children and adults. In recent years, one agent, Norwalk virus, has been shown to be responsible for about 40 percent of these disease outbreaks in the United States. Other Norwalk-like viruses have also been discovered such as Hawaii agent and Snow Mountain agent, and although they have not been well studied epidemiologically, they are likely to be responsible for many more epidemic cases of this illness.

The second clinical entity is usually sporadic, occasionally epidemic, and occurs predominantly in infants and young children (1). However, as noted below it can occur in adults. This form of illness typically produces severe diarrhea that commonly lasts for five to eight days and is usually accompanied by fever and vomiting. Rotavirus, which was discovered during the 1970's, is responsible for nearly one half of the cases of this clinical entity requiring hospitalization. Although the major target of rotavirus is the very young, it can produce surprisingly severe clinical disease in adults (1,2).

Despite the frequency of viral gastroenteritis syndromes, the etiology of these illnesses remained obscure until the 1970's. The principal investigator began his studies into the etiology and pathogenesis of this illness in 1970, with the initial goal of development of materials and methodology necessary for an understanding of this disease. Initially, he transmitted enteritis to healthy adult volunteers by the oral administration of bacteria-free, toxin-free stool filtrates derived from several outbreaks of the disease. These studies led to the discovery of the first agent responsible for viral diarrhea, Norwalk virus (3).

Norwalk virus is the prototype for a group of small, poorly defined agents. It is currently noncultivable in vitro and not disease producing for experimental animals, and was initially described by the investigator and colleagues as a small lipid-free virus (4). It was later visualized in infectious stool filtrates and partially characterized by immune electron microscopy (IEM) and ultracentrifugation (5). Other 27 nm sized Norwalk-like viruses, such as Hawaii and Snow Mountain viruses, have been uncovered by similar techniques but appear to be immunologically distinct based on IEM studies (1).

The investigators have shown that the Norwalk and Hawaii agents both produce a mucosal lesion of the proximal small intestine, the likely site for

replication of these viruses (6,7). This lesion is accompanied by transient small intestinal malabsorption, and also by delayed gastric emptying despite normal gastric morphology and secretory function (8).

The investigator has also established that clinical immunity to Norwalk virus in volunteers is novel and fails to fit immunologic concepts traditionally associated with common human viral illnesses (9): pre-existing serum antibody is paradoxically associated with the development of illness in volunteers, and lack of pre-challenge antibody is found in volunteers who remain well after exposure to the virus and also fail to seroconvert to the agent (9-11). In addition, antibody to Norwalk virus in prechallenge intestinal fluids has been found predominantly in those volunteers who subsequently developed illness. At least two forms of clinical immunity exist for Norwalk virus: one group of subjects (persistently lacking antibody) maintains long-term immunity to the virus as shown by lack of illness after initial challenge and after rechallenge up to 34 months later. A second group of volunteers (persistently possessing antibody) is susceptible to infection both upon initial exposure and again upon rechallenge 27 to 42 months later. Short term immunity exists to the virus when ill subjects are reexposed after 6 to 14 weeks.

Investigators at the National Institutes of Health, as well as the principal investigator, have developed a radioimmunoassay (RIA) technique for the detection of Norwalk virus in diarrheal stools and for quantitation of antibody to the agent (10,11). The RIA provides a laboratory handle for studies to cultivate the virus in vitro. The principal investigator has used the RIA to study forms of clinical immunity to Norwalk virus (11) (also see the preceding paragraph), and to show that Norwalk RIA serum antibody prevalence levels rise during adolescence in the United States (11). It has also been observed that antibody to Norwalk virus is acquired at a significantly earlier age in less developed and tropical areas than in more developed and nontropical areas (12,13). The RIA test has also been used to show that Norwalk virus is responsible for approximately 40 percent of viral gastroenteritis epidemics that occur in the United States (14). The principal investigator has also developed an RIA test for IgM antibody to Norwalk virus (15). This test indicates that, with volunteer sera, virus-specific IgM is not necessarily indicative of primary infection with Norwalk virus inasmuch as reinfection produces enhancement of the IgM response. Furthermore, these IgM responses in volunteers appear not to be associated with subclinical illness.

It should be noted that the RIA test for Norwalk virus and its antibody is currently available in only a few research laboratories including that of the principal investigator. This is because the procedure requires the use of precious limited human volunteer materials (stools and sera). The Norwalk RIA procedure permits the large-scale rapid testing of clinical specimens from individuals for evidence of infection with Norwalk virus. Its drawbacks, however, are the requirement for radioactive reagents and a low efficiency in detecting virus in stools from natural disease outbreaks. Greater efficiency in detecting Norwalk virus antigen in stools has been achieved in our development of an enzyme-linked immunoassay (EIA) for Norwalk virus (16,17). Epidemiologic studies have shown the importance of Norwalk virus in various parts of the world, including its involvement in waterborne, foodborne, and shipborne outbreaks of acute gastroenteritis (1). A major role of this virus in producing widespread outbreaks in the U.S. of clam and oyster associated

gastroenteritis has been shown (18) by our research group and collaborators. In addition, the investigator and colleagues have shown Norwalk virus to be a cause of travelers' diarrhea in Mexico and Thailand (19,20).

During the past decade, a second viral enteric pathogen of man has been identified and is now known to be the major cause of diarrhea in infants and young children (21,22). It can also produce illness in adults (2). This pathogen, rotavirus, has been identified by electron microscopy in stool filtrates derived from ill individuals. Serologic assay techniques have been developed for this agent by our laboratory and others to detect antibodies in human sera (23). In addition, rotavirus has been identified by our laboratory and others in diarrheal feces by RIA or enzyme-linked immunosorbent assay (EIA) techniques (23,24). Further, we have developed a monoclonal antibody based RIA technique to enhance the sensitivity and specificity of detection of rotavirus (25). We have used immunoassay methodologies to establish the role of rotavirus in diarrhea in several nations around the world, including travelers' diarrhea experienced by U.S. military populations overseas (19,26-30). During 1981, Japanese scientists successfully cultivated human rotavirus in rotated cell cultures by incorporating low concentrations of trypsin into the culture medium. This finding has been confirmed by many laboratories; although the in vitro cultivation of this virus is relatively inefficient, it has aided in the characterization of the molecular genetic properties of the virus.

Studies reveal that mechanisms of clinical immunity to rotavirus are complex (1). It seems likely that serum antibody to the virus is associated with protection from illness, and perhaps, local intestinal tract antibody as well. However, interpretation of studies is complicated by the presence of at least four human serotypes and two (perhaps three) subgroups of the virus (1). Immune responses are heteroserotypic and heterosubgroup in nature, and various human and animal rotaviruses are closely related both by serotype and subgroup. A European group has administered calf rotavirus as a potential immunogen to adults and children (31). This "vaccine" has demonstrated homotypic immunogenicity with lesser degrees of heterotypic responses, and has shown some protective clinical effects in field trial of children in developed areas who were later naturally exposed to wild rotavirus. Rhesus rotavirus vaccine has been shown to be more immunogenic and protective in third world settings than the bovine strain, and a suitable dose is being sought that eliminates concurrent side effects (32).

During the past few years, several other potential agents of viral gastroenteritis have been described, including enteric adenovirus, calicivirus, and astrovirus (1). It is important that these agents be studied to ascertain their relevance in human diarrheal disease. Among these agents, the evidence currently seems strongest that "enteric" adenoviruses are medically important pathogens like rotavirus and Norwalk virus. These adenoviruses differ from the well characterized conventional serotypes of adenoviruses which are readily propagated in standard tissue cultures and are not commonly associated with gastroenteritis. The "enteric" adenoviruses are recognized by electron microscopy in stools and cultivatable inefficiently in an adenovirus transformed cell line, Graham 293 (33). Two "enteric" serotypes (types 40 and 41) have been identified and in a limited number of studies performed to date, have been highly associated with gastroenteritis in infants and young children and much less commonly found in asymptomatic children (1,34). The potential

role of enteric adenoviruses in travelers' diarrhea or in disease in adults has not been studied. Convenient and specific immunoassays to detect enteric adenoviruses have been greatly needed, and would permit an understanding of their epidemiology as has already occurred with the use of immunoassays to study rotavirus and Norwalk virus. Some of the work accomplished during the current contract year has been directed towards development of a sensitive and specific EIA to detect enteric adenoviruses in stool specimens.

Caliciviruses have also been associated with diarrheal disease in humans (1,35). These agents are currently detected solely by electron microscopy and more convenient assays for their detection are needed so that their epidemiology can be studied. Norwalk virus possesses a single structural protein, characteristic of a calicivirus (36), and the two agents are of similar size and general shape (albeit, differing somewhat in virion surface structure). Thus, the possibility of relatedness between these two enteric viruses exists and has been studied by us during the current contract year.

Astroviruses, like caliciviruses, are small (27-35nm in diameter) and have been identified by electron microscopy in the stools of some patients with gastroenteritis (1,37). Astroviruses have been reported to be cultivatable in cell culture (38,39). However, simple diagnostic procedures have not been developed. Thus, the extent of the role of astroviruses in human diarrheal disease is not known. During the current contract year, initial efforts have been undertaken to develop procedures for convenient, specific, and sensitive detection of astroviruses.

DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO ENTERIC ADENOVIRUS TYPES 40 AND 41

Monoclonal antibodies specific for the two adenovirus serotypes (types 40 and 41) associated with gastroenteritis were prepared in the hope that they would provide badly needed specific reagents for the detection of these agents in human clinical materials. Monoclonal antibodies to the adenovirus group hexon antigen have been reported previously (40) but will react with all non-enteric adenoviruses as well as enteric adenoviruses 40 and 41, and therefore are of no specific value for the diagnosis of gastroenteritis due to enteric adenovirus. Thus, because types 40 and 41 are the only adenoviruses that have been consistently associated with gastroenteritis, specific antibodies for these types are essential for their identification.

Using a modification of the procedure described by Lee et al (41), hybridomas secreting monoclonal antibodies were prepared to each of the enteric adenoviruses types 40 and 41. Three different hybridoma cell lines were selected which produced antibody found to react by radioimmunoprecipitation with adenovirus hexon antigens. One was specific for adenovirus 40, another for adenovirus 41, and a third one reacted with both types. When tested in an enzyme immunoassay against all 41 known human adenovirus types, the type-specific monoclonal antibody against adenovirus 40 reacted homotypically, as did the monoclonal antibody against adenovirus 41. In addition, these monoclonal antibodies neutralized the homologous enteric adenovirus type. The monoclonal antibody which reacted with both enteric adenovirus types also showed lower levels of reactivity with the Group C adenoviruses types 2, 5, and 6. Thus, the monoclonal antibodies produced provided a definitive means for rapid identification of specific enteric adenovirus serotypes.

A summary of the characteristics of the monoclonal antibodies produced is provided in the following table.

Table 1. Characteristics of monoclonal antibodies to enteric adenoviruses

Hybridoma designation	Antibody specificity	Antibody class	Virion	
			reactive site	Virus neutralization
1C11	Ad40	IgG2a	hexon	yes
9D4	Ad40,41,2,5,6	IgG1	hexon	no
9B9	Ad41	IgG2a	hexon	yes

These data have been accepted for publication in the Archives of Virology (42).

ANTIGEN DETECTION WITH MONOCLONAL ANTIBODIES FOR DIAGNOSIS OF ENTERIC ADENOVIRUS GASTROENTERITIS

Efforts were next undertaken to utilize the enteric adenovirus-specific monoclonal antibodies in an EIA format for the detection of adenovirus 40 and 41 infections in stool specimens from patients with gastroenteritis. The sensitivity of the EIA was compared to isolation of virus in Graham 293 cells from stools which contained particles of adenovirus morphology by electron microscopy. The standard for specificity was analysis of adenoviral genome profiles after digestion with SmaI endonuclease. As shown in the following table, the sensitivity was 95.8% (23/24) for Ad40 and 97.1% (34/35) for Ad41. The specificity was 95.7 (45/47) and 97.2% (35/36) respectively.

Table 2. Type specific detection of adenovirus types 40 and 41 directly in stool specimens.

Monoclonal antibody designation/specificity	EIA results	
	% Sensitivity [*]	% Specificity ⁺
1C11/Ad40	95.8 (23/24)	95.7 (45/47)
9B9/Ad41	97.1 (34/35)	97.2 (35/36)

* Sensitivity of the EIA was compared to isolation of virus in Graham 293 cell culture

⁺ Typing of virus for specificity determinations was done by analysis of DNA restriction endonuclease digests.

The two type-specific monoclonal antibodies could be mixed in an EIA to identify enteric adenoviruses in stools without loss of reactivity in either type. Thus, these monoclonal antibody based EIA tests provide an effective and convenient means for rapid diagnosis and type-specific identification of enteric adenoviruses in gastroenteritis, and will substantially facilitate epidemiological studies of their infection. These data have been accepted for publication in the Journal of Infectious Diseases (43).

It is important to reemphasize the need for specificity in detecting adenovirus in stools as being due to types 40 and 41, because other adenovirus serotypes can be shed in the stools of asymptomatic persons or those ill who are not experiencing gastroenteritis. All adenoviruses, regardless of serotype, can be detected in stools using a group reactive polyclonal or monoclonal antibody-based assay. These group antigen-reactive tests, however, fail to differentiate enteric types from non-enteric types.

The monoclonal antibody based EIA tests specific for enteric adenoviruses 40 and 41 have now permitted us to initiate studies of their role in diarrheal disease among a variety of populations and age groups. We have decided to initiate studies involving populations in the third world because what is currently known in detail about the epidemiology of enteric adenoviruses is based solely on the study of populations in temperate climates. One population studied thus far in collaboration with Dr. H. DuPont, U. Texas, Houston, is U.S. students who have traveled to Mexico during the summer months and developed diarrhea. We have tested 112 diarrheal stool samples, and all were negative for enteric adenoviruses. We plan to test additional specimens from student travelers in the fall and winter seasons, in which enterotoxigenic *E. coli* infections are less common than in the summer. In addition, we have initiated a study of 2060 pediatric stools collected by Drs. P. Echeverria, D. Taylor and colleagues of the Armed Forces Research Institute of Medical Sciences, Bangkok. Specimens were collected from children under age five over

a one year period at an outpatient clinic in Bangkok. 1112 children with gastroenteritis and 948 children without this syndrome are being studied. With the testing of approximately one-third of the samples to date, no clear-cut differences have appeared in the presence of enteric adenoviruses among sick and well children.

ANTIGENIC RELATIONSHIPS AMONG HUMAN CALICIVIRUSES AND NORWALK VIRUS

Because of morphological and biochemical similarities (see Background Information section) between Norwalk virus and human caliciviruses (HCV), we have sought evidence for immunological relatedness among these viruses. These studies were performed in collaboration with two investigators with expertise with human calicivirus, Drs. David Cubitt of London, England and Shunzo Chiba of Sapporo, Japan. Electron microscopic and immune electron microscopic (IEM) assays to study HCV and its antibodies were utilized in England, and radioimmunoassays (RIA) were employed for HCV antigen and antibody in Japan. Radioimmunoassays for Norwalk virus antibody titers were performed in our laboratory.

Our collaborative data can be summarized as follows: three serologically distinct HCV strains, (UK1, UK2, Japan) were demonstrated by IEM, as was evidence for two additional strains (UK3, UK4). Although HCV strains and Norwalk virus were distinct by IEM, 12 of 20 patients with gastroenteritis due to HCV UK4 and 2 of 8 due to UK2 showed seroconversions to Norwalk virus by RIA. These naturally occurring human RIA antibody responses strongly support the concept that Norwalk virus belongs to the family Caliciviridae. An RIA test for HCV Japan antigen also detected HCV UK1, UK2 and UK4, and thus appears to identify a group specific antigen for these viruses. An RIA test for antibody to HCV Japan failed to identify seroconversions in 45 of 47 HCV UK cases. These results may reflect different reactivities of various immunologic tests for the identification of infections due to small gastroenteritis viruses.

The following table details the seroconversions to Norwalk virus detected in symptomatic patients with calicivirus infection.

Table 3

Seroconversion to Norwalk Virus and
Human Calicivirus Among Symptomatic Patients
In Gastroenteritis Outbreaks Due to Calicivirus

<u>Outbreak/HCV Strain</u>	<u>Percent Seroconversion</u> [*]	
	<u>Norwalk Virus</u>	<u>Calicivirus</u>
Tower Hamlets/UK4	59 (10/17)	76 (13/17)
Colchester/UK4	67 (2/3)	100 (3/3)
Harefield/UK2	67 (2/3)	100 (3/3)
Portsmouth/UK2	0 (0/5)	100 (5/5)
Shenley/UK1	0 (0/1)	100 (1/1)

* Seroconversion (4 fold or greater rise in antibody titer) to Norwalk virus measured by RIA and Seroconversion to Calicivirus measured by IEM, using homologous virus strain

The Tower Hamlets UK4 outbreak provides evidence for at least a one-way serological cross-relatedness between Norwalk virus and human calicivirus UK4. Further support for this relatedness was seen in the study of a UK4 outbreak in Colchester in which two of three ill persons seroconverted to Norwalk virus. It should be noted that the Norwalk RIA antibody titers of patients with calicivirus infection in the Tower Hamlets and Colchester outbreaks who showed seroconversion to Norwalk virus were of a magnitude similar to those that are observed in well-defined outbreaks of Norwalk disease.

Fifteen serum pairs were also evaluated from asymptomatic persons in the Tower Hamlets outbreak, all of whom remained well presumably due to their failure to ingest contaminated oysters which were felt to have been the vehicle by which infection was initiated (data not shown). One of these 15 persons seroconverted to Norwalk virus, a different individual seroconverted by IEM to the homologous calicivirus.

Seroconversion to Norwalk virus developed in two of eight ill patients from HCV UK2 outbreaks in Harefield and Portsmouth. Two of three Harefield individuals seroconverted to Norwalk virus as compared to none of five Portsmouth patients. All eight persons seroconverted to the homologous HCV strain by IEM.

Thus, the data indicate that there may be a difference in the degree of

antibody reactivities to Norwalk virus antigen between sera collected from calicivirus outbreaks due to different viral strains: 12 of 20 (60%) patients exposed to HCV UK4 seroconverted to Norwalk virus as compared with 2 of 8 (25%) patients in UK2 outbreaks and 0 of 1 patient in a UK1 outbreak. Portions of these studies have recently been published in a CIBA Foundation Symposium volume (44) and all data have recently been submitted for publication.

EVIDENCE FOR IMMUNOLOGICAL RELATEDNESS BETWEEN SNOW MOUNTAIN AGENT AND NORWALK VIRUS

As noted in the Background Information section, the Snow Mountain agent is a 27nm sized "Norwalk-like" agent that, like Norwalk virus, is derived from an outbreak of epidemic gastroenteritis and can produce disease in volunteers. As part of a laboratory evaluation of a Snow Mountain agent disease outbreak in which 6 of 11 ill students seroconverted by EIA to the Snow Mountain agent, two of 21 affected patients also seroconverted by RIA to Norwalk virus (in addition to Snow Mountain). These findings may be connected to our observation (in the preceding section) of HCV relatedness to Norwalk virus by RIA but not by IEM tests using human sera. This finding may be explainable by different antigens detected by the two assays. The IEM technique measures antigens on the surface of the virion and IEM cross-reactions between viral strains likely reflect very close serotypic relatedness or identity. However, the Norwalk virus RIA likely produces a broader serological reactivity with inner or soluble components of the virion contained in human stool. Thus, the Norwalk RIA procedure can be expected to detect broad immunological reactions between virus strains, and in this way probably has also demonstrated that Norwalk virus and Snow Mountain agent share some antigenic determinants. The Snow Mountain-Norwalk seroconversion data were published during the current contract year in Pediatrics (45).

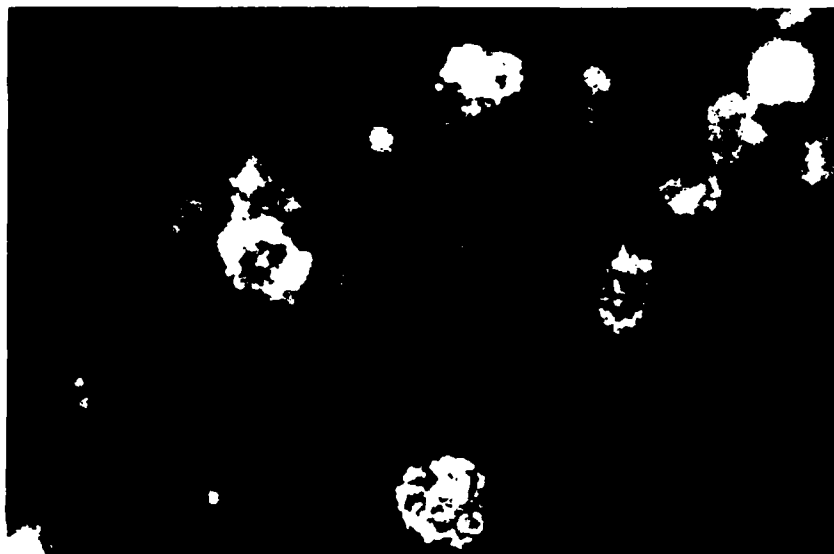
IN VITRO CULTIVATION STUDIES WITH HUMAN ASTROVIRUS AND CALICIVIRUS

It is reported that human astrovirus and calicivirus can be cultivated in vitro, albeit inefficiently, with the aid of trypsin (38,46). We have sought to confirm these observations, in order to be able to generate purified virus particles as an immunogen for the preparation of highly specific monoclonal antibody reagents against astrovirus and calicivirus. These reagents would enable us to develop rapid, sensitive, and specific immunoassays for determining the epidemiological importance of these human gastroenteritis viruses in diarrheal disease, similar to what we are currently doing with the enteric adenoviruses.

Dr. John Kurtz (Oxford, England) has kindly provided us with the five human astrovirus serotypes that he has described, and has also provided us with hyperimmune rabbit sera against each type. Using his astrovirus culture methodology in LLCMK2 and HEK cells (38), we have been able to confirm the growth of astrovirus types 1,2,4 and 5, using 10ug/ml of trypsin in serum-free culture media. Types 4 and 5 replicate to date only in HEK cells, whereas types 1 and 2 grow in both HEK and LLCMK2 cells. Astrovirus type 3 has not been able to be passaged in either cell culture. Astrovirus types 1,2 and 5 have been serially passaged 20 to 30 times each, and type 4 ten times. Virus has been detected by electron microscopy in culture passages. Also, the presence of virus antigen in the cytoplasm of infected cells can be readily shown by immunofluorescent staining using Kurtz's type-specific rabbit anti-

sera. An example of immunofluorescence obtained with astrovirus type 2 infection in LLCMK2 cells is shown in the accompanying Figure.

Figure 1
Immunofluorescent Staining of Astrovirus Type 2 Infected
LLCMK2 cells.



Dr. David Cubitt (London, England) has provided us with several human calicivirus (HCV) positive stools for attempted in vitro cultivation, as well as human sera known to possess IEM antibody to HCV. Using his calicivirus culture methodology in HEK and dophin kidney cells (46), we have been unable to confirm the in vitro cultivation of HCV. We have looked for immunofluorescence using the sera provided to us by Dr. Cubitt.

ANTIGENIC RELATIONSHIPS AMONG HUMAN ASTROVIRUS SEROTYPES

The ability to cultivate four astrovirus serotypes has enabled us to obtain sufficient quantities of antigen to assess potential antigenic relatedness between astrovirus serotypes. Using Kurtz's polyclonal rabbit antisera, we have confirmed the type-specificity of reactions by immunofluorescence,

that is, type 1 antiserum reacts with type one virus only and so forth. However, we are observing cross-reactions between serotypes when infected cells are examined by EIA (rather than immunofluorescence). The EIA test that we have developed uses Kurtz's polyclonal rabbit sera against individual astrovirus serotypes and detects an astrovirus group antigen inasmuch as antiserum raised against one serotype reacts with the other types by EIA. Thus, we have reagents available that are type-specific for astrovirus in one immunological assay and group-specific for these viruses in another assay. It is now clear that astroviruses possess a group antigen, and this information should be able to be exploited in the development of EIA tests for the detection of astrovirus antigen in stool specimens.

DEVELOPMENT OF MONOCLONAL ANTIBODIES AGAINST HUMAN ASTROVIRUS

Based on our observation that astroviruses share a common group antigen (see preceding section), we have initiated studies to prepare monoclonal antibodies to astrovirus with particular attention being paid to selecting astrovirus group reactive monoclonal antibodies. Recently, we have developed a panel of monoclonal antibodies to astrovirus type 2 antigen. This virus was cultivated, gradient purified and inoculated into mice whose spleen cells were later stimulated in vitro with purified astrovirus type 2 (procedure of in vitro immunization). Screening by EIA of hybridomas produced to date indicates that antibodies are being produced to both group specific and type specific antigens of astrovirus. Clones of each are being selected for further study. One strongly group reactive monoclonal has already been characterized as being class IgG1, and it reacts by immunofluorescence with the 4 astrovirus types that we can cultivate. It therefore appears likely that we have been able to select a specific group-reactive monoclonal antibody which holds promise for facilitating epidemiological studies on the role of astroviruses in human viral gastroenteritis.

COLLABORATIVE EPIDEMIOLOGICAL STUDIES WITH THE MILITARY

Three collaborative studies on the role of viral agents in gastroenteritis have been performed with scientists in the U.S. military during the current contract year. The first study is described above and represents a collaboration with Dr. P. Echeverria of AFRIMS, Bangkok designed to assess the role of enteric adenoviruses in outpatient pediatric diarrhea.

A second study was performed with Major David Smith and colleagues, Division of Preventive Medicine, Walter Reed Army Institute of Research. This collaboration was designed to ascertain the potential role of Norwalk virus in acute diarrhea experienced by U.S. Army troops on maneuvers in Panama. Twenty-two diarrheal stools and 57 acute and convalescent serum pairs were sent to us for analysis. All 22 stools were negative for Norwalk virus by EIA. However, 12 of 57 serum pairs (21%) seroconverted by RIA to Norwalk virus, indicating evidence for some Norwalk virus related diarrhea in the study sample.

The third study was performed in collaboration with the Naval Medical Research Institute Detachment in Lima, Peru (Drs. C.H. Gardiner, J. Escamilla, I.A. Phillips, M. Kilpatrick) and Naval Medical Research Institute in Bethesda (Dr. A.L. Bourgeois). This study represented a comprehensive six month evaluation of diverse etiologic agents for travelers' diarrhea encountered by

U.S. Navy and Marine Corps personnel visiting major seaport cities of Central America, South America and West Africa. Not unexpectedly, a diversity of enteropathogens was encountered, with enterotoxigenic E. coli accounting for one-third of cases. Norwalk virus detection and serological studies, performed by our laboratory indicated that 12 per cent of all cases (137) of diarrhea were associated with Norwalk virus or an immunologically closely related agent ("Norwalk-like" virus). Most Norwalk illness occurred as a common source outbreak during a Navy-Marine luncheon held in Guayaquil, Ecuador, consisting of raw fish, salad and meats. These findings are consistent with published outbreaks of Norwalk disease. The data are being prepared for publication by the Lima group.

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PUBLICATIONS OF WORK SUPPORTED BY THIS CONTRACT

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